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Cloning of a mRNA Preferentially Expressed in Chondrocytes by Differential Display-PCR from a Human Chondrocytic Cell Line That Is Identical with Connective Tissue Growth Factor (CTGF) mRNA

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Chondrocyte- or chondrosarcoma cell line (HCS)-specific DNA fragments were obtained using differential display-PCR. Nucleotide sequences of 32 species derived from HCS cells were determined. One of the sequence tags (tag no. 24) corresponded to the nucleotide sequence of connective tissue growth factor (CTGF). Northern blot analysis showed that CTGF was highly expressed in HCS cells and rabbit growth cartilage cells in culture but was not expressed in osteoblastic cells in culture. *In situ* hybridization revealed that CTGF was expressed only in the hypertrophic chondrocytes of costal cartilage and the vertebral column in embryonic mice. The expression of CTGF in HCS cells was up-regulated by the addition of TGF-β or BMP-2. These findings suggest that CTGF participates in endochondral ossification. © 1997 Academic Press

In the process of endochondral ossification, resting cartilage cells first differentiate into growth cartilage cells which actively proliferate. These growth cartilage cells mature and produce much extracellular matrix. Then the cells become hypertrophic and the matrix is mineralized; finally, the cartilage is replaced by bone (1).

During these processes, many kinds of hormones such as parathyroid hormone and active form of vitamin D_3 (2-5), vitamins such as ascorbic acid and retinoic acid (6-8), and growth factors such as IGFs, FGF and TGF-β (9-13) have been shown to regulate the proliferation and differentiation of chondrocytes. In addition, BMP is also an important factor for endochondral bone formation (14). Furthermore, novel BMP-like proteins such as CDMP-1 and -2 have recently been reported as cartilage-forming proteins (15). However, many of these factors are widely distributed in many tissues and little is known about the molecular mechanism of their chondrocyte-specific regulation. Therefore, other factors which specifically regulate the function of chondrocytes may exist.

By the method of differential display of mRNA, mRNA derived from many different tissues or from tissues of different developmental stages can be compared, and specifically expressed cDNA fragments can be isolated (16). In this study, we isolated chondrocyte-specific sequence tags from newly established immortal, clonal chondrocytic cell lines (HCS) (17-21) by the differential display-PCR method and analyzed the expression of one sequence tag (tag no. 24) corresponding to connective tissue growth factor (CTGF) (22-24).

MATERIALS AND METHODS

Cell culture. Human chondrosarcoma-derived chondrocytic cell lines (HCS-2/8, HCS-2/A) (6, 17-21), and human osteosarcoma cell line (Saos-2) (25) were cultured in DMEM containing 10% fetal bovine serum (FBS). Rabbit growth cartilage cells (RGC) and resting cartilage cells (RRC) were isolated from growth and resting cartilage of ribs of young rabbits (7). Mouse osteoblastic cells (MC3T3-E1) (26, 27) were cultured in αMEM containing 2% FBS. To investigate the effect of cytokines on HCS-2/8 cells, human recombinant TGF-β (kindly supplied by Takara Shuzo Co. Ltd.) and human recombinant BMP-2 (kindly supplied by Yamanouchi Pharmaceuticals) were added to subconfluent cultures of the cells.

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Abbreviations used: IGF, insulin-like growth factor; FGF, fibroblast growth factor; TGF, transforming growth factor; BMP, bone morphological protein; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; HCS, human chondrosarcoma-derived chondrocyte; CTGF, connective tissue growth factor; MEM, minimum essential medium; DMEM, Dulbecco's minimum essential medium; ALPase, alkaline phosphatase; RGC, rabbit growth cartilage; RRC, rabbit resting cartilage.

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Differential display of mRNA and isolation of CTGF cDNA. Total RNA was isolated from confluent cultures of these cells according to the method of Chomczynski et al. (28) and treated with DNase I. For differential display-PCR, three kinds of anchor primers and three kinds of arbitrary primers were used. Their nucleotide sequences are as follows: 5'-CCCGGATCC$(T)_{15}$ N-3' (N: A, C or G) (anchor primers); 5'-GGACTAAGCTTCAGCATTCCC-3'(NGF); 5'-GTGAGAAGCTTGATG-ACCATCC-3' (BDNF); 5'-GGTGAACAAGGTGATGTCCATC-3' (NT-3). After the amplification, the reaction mixture was applied to acrylamide gels (6%) and DNA fragments were extracted from the gels. Re-amplified DNA fragments were sequenced directly or after subcloning into appropriate plasmid vectors. CTGF cDNA was isolated from cDNAs which were synthesized from poly(A)$^+$ RNA isolated from total RNA described above by PCR method using the consensus sequence in CTGF family (CCN family) cDNAs as primers.

Northern blot analysis. Total RNA isolated from the cells and cell lines described above and total RNA isolated from various rabbit tissues by the same method (28) were used for Northern blotting. In the cases of HCS cells, rabbit chondrocytes and cartilage tissues, treatment with 200 μg/ml of proteinase K (Gibco BRL) was introduced at the final step of total RNA preparation. After treatment with DNaseI, the RNA was applied on 1% denatured agarose gel. For hybridization, radiolabeled probes were prepared by RT-PCR with two specific primers in tag no. 24 [5'-TTGTAGCTGATCAGT-CTTTCCAC-3'(DD24); 5'CAACTAAAAAGGTGCAAACATGTAA-3' (DD24R2)].

In situ hybridization. As a riboprobe, digoxigenin -11-UTP-labeled RNA was prepared from the cDNA fragment of CTGF using a DIG RNA labeling kit (Boehringer Mannheim GmbH). Hybridization was performed following the methods of Noji et al(29). Hybridized riboprobes were detected using DIG nucleic acid detection kit (Boehringer Mannheim GmbH) following the manufacturer's protocol.

Reverse transcriptase (RT)-PCR for quantitation of CTGF mRNA expression. A set of primers specific for tag no. 24 described above (DD24 and DD24R2) which amplified a 120 bp DNA fragment of the cDNA was used for amplification. PCR products were analyzed by agarose gel electrophoresis. For the analysis of expression level of aggrecan core protein mRNA, a set of primers which amplified a 303-bp DNA fragment of the cDNA was constructed. Their nucleotide sequences were as follows: 5'-CGCGAGACCTGGGTGGATGC-3' (AGGS); 5'-GAAGGGG/CAG-G/CTGGTAATTGC-3' (AGGAS).

Measurement of alkaline phosphatase (ALPase) activity. Normal growth chondrocytes cultured in 48-well microplates (αMEM containing 10% FBS) were collected in 0.5 M Tris (pH 9.0) containing 0.9% NaCl and 1% Triton X-100. Then the cells were homogenized and ALPase activity in the resultant supernatants was determined by the method of Majeska and Rodan (30).

RESULTS

Isolation and characterization of CTGF. By differential display PCR, we isolated about 30 species of DNA sequence tags, which were expressed selectively in chondrocytes, and especially highly expressed in HCS-2/8. Comparison of the results of sequence analysis to the sequences in the DNA sequence data bases revealed 10 sequences with similar mitochondrial DNA or cDNA, 7 novel DNA sequences with poly(A)$^-$ stretch, and several sequences which showed high homology with known cDNA sequences (Table 1). There was one sequence tag (tag no. 24) which showed high homology with connective tissue growth factor (CTGF) (Table 1). Its nucleotide sequence showed high homology with the sequence of the 3'-terminal region of CTGF cDNA, and the nucleotide sequence and the amino acid sequence of coding region of the cloned cDNA which contained tag no. 24 was completely the same as that of human CTGF except for two nucleotides (two amino acids) (data not shown). We, therefore, concluded that the cDNA containing tag no. 24 was CTGF. The difference in nucleotide sequence and in amino acid sequence between the two cDNAs might have occurred during the DNA amplification step because we found several other clones which showed a CTGF sequence in those positions.

Northern blot analysis. The expression level of CTGF mRNA was analyzed by Northern blotting using PCR products amplified with specific primers as a probe (Fig. 1). As shown in Fig. 1A, CTGF mRNA was highly expressed in HCS-2/A and 2/8, and was also expressed in rabbit normal growth cartilage cells. Little or no expression was observed in osteoblastic cells or osteosarcoma cells. Among various rabbit tissues, CTGF mRNA was highly expressed only in growth cartilage and slight or almost no expression was observed in other tissues (Fig. 1B).

In situ hybridization. To analyze the expression of CTGF mRNA in tissues, *in situ* hybridization was per-

TABLE 1

Sequence Homologies of Isolated DNA Fragments

Sequence	% homology	Clone
Unknown		2, 4, 11, 15, 21, 30, 32
hm-, rb-mitochondrial DNA	81.2–98.0	1, 3, 10, 13, 14, 20, 22, 23
hm-novel DNA-binding protein	83.9–91.6	7, 16, 17, 25, 26
hm-mitochondrial cytochrome *b* oxidase	98.1	6
hm-ribosomal protein S11	82.4	8
hm-arginine-rich nuclear protein	94.1	18
hm-mitochondrial cytochrome *c* oxidase	70.6	19
hm-connective tissue growth factor	88.7	24 (tag no. 24)
hm-interferon α/β receptor	60.2	28
hm-apolipoprotein D	72.1	29

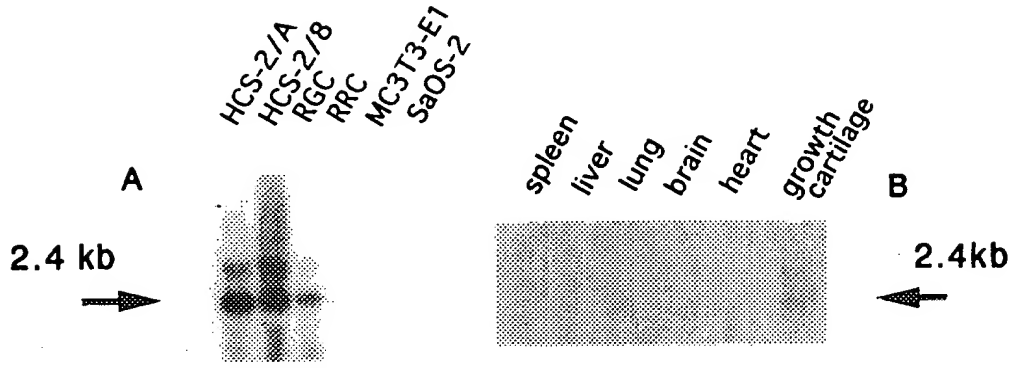

FIG. 1. Northern blot analysis of the expression of CTGF mRNA in various cell lines and various tissues. (*A*) About 15 μg of total RNA was used for the detection of CTGF mRNA in chondrocytic cells (HCS-2/A, HCS-2/8, RGC, and RRC) and osteoblastic cells (MC3T3-E1 and Saos-2). (*B*) About 12 μg of total RNA was used for the detection of CTGF mRNA in various rabbit normal tissues including growth cartilage. Arrows indicate the position of CTGF mRNA.

formed using tissue sections of mouse embryo (E17). As shown in Fig. 2, CTGF mRNA was specifically detected in the hypertrophic regions of costal cartilage (Fig. 2 A,C) and the vertebral column (Fig. 2 B,D). In the pattern of costal cartilage (Fig. 2A), an intense signal was observed in hypertrophic chondrocytes (closed arrowhead) and almost no signal was observed in chondrocytes in the proliferating zone (open arrowhead).

Change in expression of CTGF mRNA in rabbit growth cartilage cells in culture. We next analyzed the expression level of CTGF *in vitro* in the continuous cultures of rabbit growth cartilage cells by RT-PCR using the set of specific primers described above. The expression of CTGF was increased during the continuous culture of growth cartilage cells. The highest level (about four times higher than that in initial stage) was

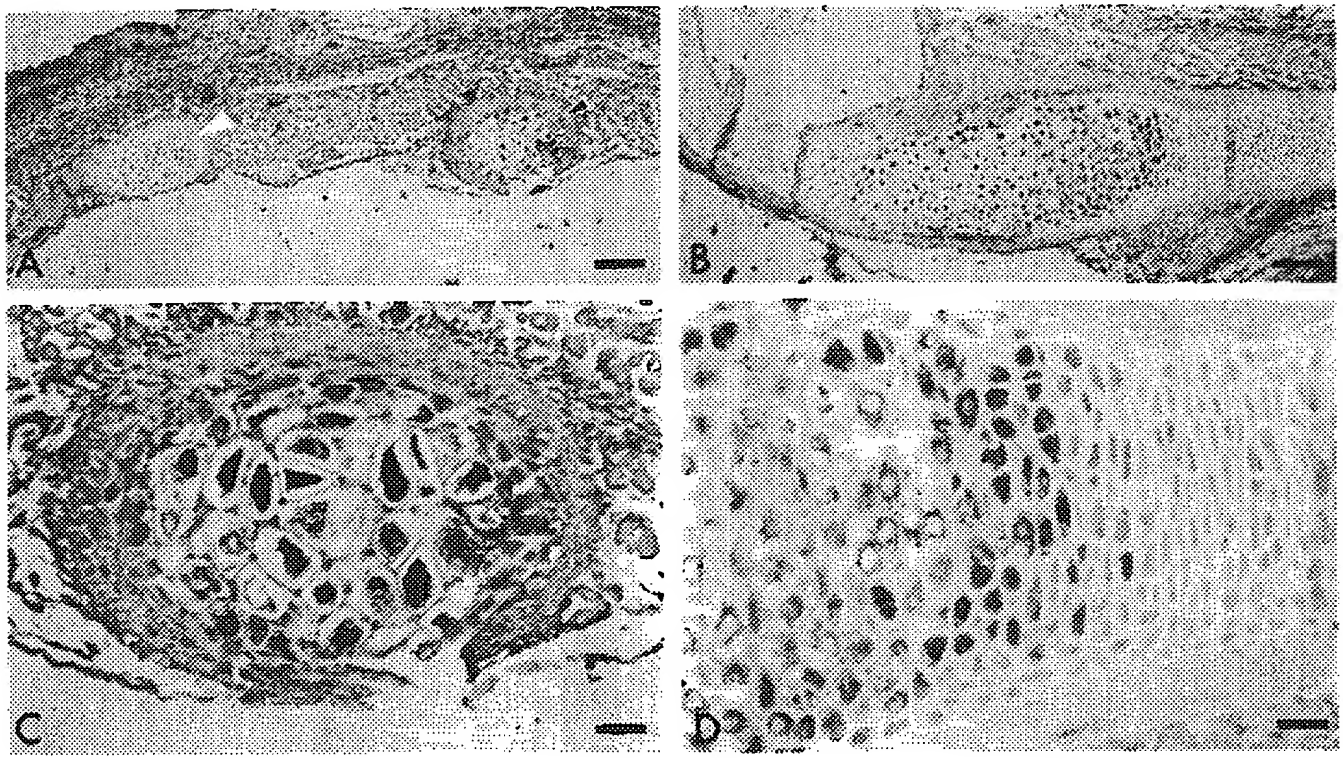

FIG. 2. Analysis of the expression patterns of CTGF mRNA by *in situ* hybridization. Tissue sections of mouse embryo (E17) were used for hybridization. Hypertrophic region [closed arrowhead in (*A*)] and a higher magnification of hypertrophic region (*C*), and proliferative zone [open arrowhead in (*A*)] of costal cartilage. Vertebral cartilage including hypertrophic region (*B*) and its higher magnification (*D*). Bars indicate 80 μm (*A* and *B*) and 20 μm (*C* and *D*).

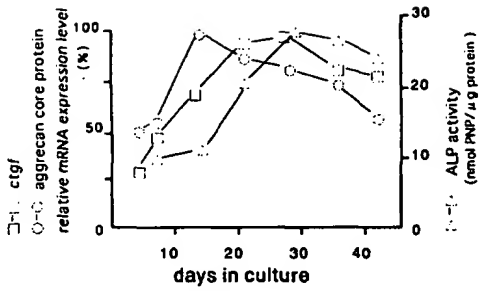

FIG. 3. Change of CTGF mRNA expression in cultured rabbit growth cartilage cells (RGC). Relative levels of the expression of CTGF mRNA (□) and aggrecan core protein (○) were quantitated by RT-PCR using the specific primers described in "MATERIALS AND METHODS" and are expressed as a percentage of the maximum level of each mRNA expression. ALPase activity in RGC (△) was indicated as nmol PNP cleaved per minute per μg cellular protein. Cells were harvested on the days indicated.

observed on day 28. In this system, the expression of aggrecan core protein mRNA reached the highest level about two weeks earlier than the expression of CTGF mRNA and alkaline phosphatase activity reached the highest level with a delay of several days compared with the expression of CTGF mRNA (Fig. 3), indicating that day 28 was in the early hypertrophic stage.

Induction of CTGF mRNA by TGF-β and rhBMP. RT-PCR revealed that TGF-β and hrBMP-2 enhanced the expression of CTGF (Fig. 4). The expression of CTGF was up-regulated 1.8 fold 12 h after the addition of 10 ng/ml of TGF-β, and was also up-regulated 2.2-fold (at 100 ng/ml) and 2.4-fold (at 300 ng/ml) after 12h of the addition of rhBMP-2.

DISCUSSION

In this study, we isolated sequence tag no. 24 corresponding to 3'-terminal region of CTGF cDNA which was observed to be expressed in chondrocytic cells but not in osteoblastic cells, indicating that the differential display method is useful for isolation of genes expressed specifically in various cells and under specific conditions.

Among 32 DNA tags, there were 7 novel DNA sequences and several DNA sequences which showed high homology with known cDNA sequences. Moreover, most genes among the later cDNA sequences such as those of CTGF, novel-DNA binding protein, arginine-rich nuclear protein and apolipoprotein D and their gene products have not been found in chondrocytes and cartilage. In addition, there were no sequence tags of well known, chondrocyte-specific genes such as aggrecan and collagen type II in our clones sequenced (Table 1). Thus, by our modified method of differential display-PCR, useless sequencing can be avoided and time and labor can be saved.

CTGF is a member of the CCN gene family which includes three subfamilies (*cef*10/*cyr*61, *ctgf*/*fisp*-12 and *nov*) (24). Recently, the *cyr*61 gene, which is an immediate early gene and is expressed in mouse 3T3 fibroblasts, was also found to be expressed in developing mouse cartilaginous elements and placental tissues (31). There are some differences in the expression pattern of *cyr*61 and CTGF. The expression of *cyr*61 was observed in newly formed cartilaginous elements and it was diminished during the development of the vertebral column of 12.5- to 15.5-day embryos (31). The *in situ* hybridization revealed a high level of expression of CTGF in hypertrophic chondrocytes of 17-day embryos (Fig. 2), and the *in vitro* culture system of rabbit growth cartilage cells also showed the highest expression of CTGF in the early hypertrophic stage (Fig. 3). Thus, both *cyr*61 and CTGF encode novel growth factors which are related to chondrogenesis, but their function may be different and complementary.

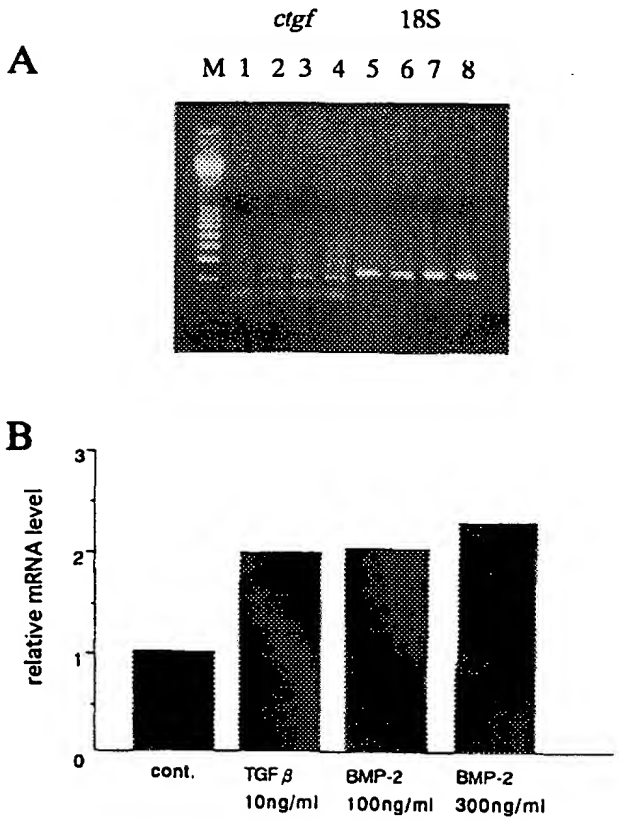

FIG. 4. RT-PCR of the expression of CTGF mRNA in HCS-2/8 cells induced by TGF-β or BMP-2. The cells were harvested 12 h after addition of TGF-β (10 ng/ml) or hBMP-2 (100 and 300 ng/ml). DNase I-treated total RNA (0.5 μg) was used for RT-PCR. (*A*) Agarose gel electrophoresis of the PCR products. M: ladder marker; 1 and 5: control; 2 and 6: TGF-β (10 ng/ml); 3 and 7: hBMP-2 (100 ng/ml); 4 and 8: hBMP-2 (300 ng/ml). (*B*) In the densitometric analysis, the amount of specific PCR products was normalized by the amount of PCR products of 18S ribosomal RNA. The ordinate indicates the ratio of treated cultures to PBS-treated control.

The stimulatory effect of CTGF mRNA expression by TGF-β and BMP-2 (Fig. 4) suggests that a relationship exists between CTGF and the formation of cartilage tissue. Because TGF-β and BMP-2 are known as bone-forming proteins and stimulate differentiation and proliferation of chondrocytes (12-14), CTGF may be a mediator of these cytokines in cartilage tissue. Jingushi et al. (32) reported that gene expression of TGF-β is highest in hypertrophic chondrocytes in the process of endochondral ossification during fracture repair. The highest expression of CTGF in hypertrophic expression might be due to the highest gene expression of TGF-β in this type of cells because hypertrophic chondrocytes as well as proliferating chondrocytes have been shown to have TGF-β receptors (33). Recently, Mason et al. (34) reported that the *twisted gastrulation* (*tsg*) gene encoding a secreted protein related to CTGF and *decapentaplegic* (*dpp*) gene, a BMP homologue cooperated in pattern formation in *Drosophila*. This finding also suggests a close relationship between CTGF and BMP. We are further analyzing the function of CTGF in the process of endochondral ossification.

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